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Chapter 5

Microvascular damage in type 1 diabetic patients is reversed in the first year after simultaneous pancreas-kidney transplantation

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Abstract

Background: Simultaneous pancreas-kidney transplantation (SPK) is an advanced treatment option for type 1 diabetes mellitus (DM) patients with microvascular disease including nephropathy. Sidestream darkfield (SDF) imaging has emerged as a noninvasive tool to visualize the human microcirculation. This study assessed the effect of SPK in diabetic nephropathy (DN) patients on microvascular alterations using SDF and correlated this with markers for endothelial dysfunction.

Methods: Microvascular morphology was visualized using SDF of the oral mucosa in DN (n=26) and SPK patients (n=38), healthy controls (n=20), DM1 patients (n=15, DM≥40ml/min) and DN patients with a kidney transplant (KTx,n=15). Furthermore, 21 DN patients were studied longitudinally up to 12 months after SPK. Circulating levels of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and soluble thrombomodulin (sTM) were measured using ELISA.

Results: Capillary tortuosity in the DN (1.83±0.42) and DM≥40ml/min (1.55±0.1) group was increased and showed reversal after SPK (1.31±0.3, $p<0.001$), but not after KTx (1.64±0.1). sTM levels were increased in DN patients and reduced in SPK and KTx recipients ($p<0.05$), while the Ang-2/Ang-1 ratio was normalized after SPK and not after KTx alone (from 0.16 ±0.04 to 0.08 ±0.02, $p<0.05$). Interestingly, in the longitudinal study reversal of capillary tortuosity and decrease in Ang-2/Ang-1 ratio and sTM was observed within 12 months after SPK.

Conclusion: SPK is effective in reversing the systemic microvascular structural abnormalities in DN patients in the first year after transplantation.

Introduction

Diabetes mellitus (DM) is a severe metabolic disease that results in macrovascular as well as microvascular complications including retinopathy, nephropathy and neuropathy (1;2). The majority of these microvascular complications is associated with endothelial dysfunction, and upregulation of different angiogenic growth factors including angiopoietin-2 (Ang-2) (2;3) and soluble thrombomodulin (sTM) release in the circulation (4;5). Negative interference of Ang-2 with Ang-1 mediated Tie-2 signaling results in disruption of perivascular stromal cell–endothelial cell interaction, subsequent vessel destabilization and abnormal microvascular remodeling (6;7).

Simultanous pancreas-kidney transplantation (SPK) has become an important option in the treatment of diabetic patients with nephropathy and seems to be superior to kidney transplantation alone (KTx), since SPK is associated with a better glycemic control as well as improved patient survival (8-10). It has been shown that SPK and pancreas transplantation alone effectively prevent the recurrence of diabetic nephropathy (DN) (11-15). However, in terms of stabilization or improvement of pre-existing neuropathy and retinopathy debate still continues on the benefit of SPK (16-20). Early non-invasive monitoring of the microvasculature may be of clinical value to assess progression of microvascular disease during diabetes and treatment efficacy after SPK. Sidestream darkfield (SDF) imaging has recently emerged as a non-invasive tool to visualize the human microcirculation and to assess microvascular remodeling (21). We have previously used this validated technique to compare the labial mucosal capillary tortuosity, as markers for microvascular disease, in diabetic patients with and without coronary artery disease (CAD) and healthy non-diabetic controls. Diabetic patients showed increased capillary tortuosity, especially in patients with CAD (22). However, to our knowledge no previous studies have used SDF imaging to monitor microvascular alterations before and after SPK.

In the present study, the effects of SPK on microvascular damage is assessed in a cross-sectional and longitudinal study using SDF imaging and correlated with markers for endothelial dysfunction. We hypothesized that SPK will reverse the microvascular damage in patients with diabetic nephropathy.

Material and Methods

Patients

All procedures were approved by the institutions Medical Ethical Committee. Written informed consent has been obtained from all the patients and healthy controls. A total of 114 persons (64 males and 50 females) were enrolled in this cross-sectional study after giving informed consent. Thirty-eight patients with simultaneous SPK were included (SPK group). Furthermore, we included 15 patients with a functioning kidney graft (KTx group), consisting of patients with a solitary kidney transplantation (n=13) and patients who received SPK and lost their pancreatic graft due to vascular thrombosis (n=2) at 2 and 4 days after transplantation. Biochemical markers for endothelial dysfunction including Ang-1, Ang-2 and sTM, together with mucosal capillary density and morphology were compared to 26 DM type 1 patients with nephropathy (DN group) on the waiting list for SPK and DM type 1 patients with an estimated glomerular filtration rate (eGFR) of ≥40ml/min (n=15, DM≥40ml/min group). Patients with active infection, liver failure, active auto-immune disease, epilepsy or malignancy in the last 5 years (except patients treated for basal cell carcinoma that were in full remission) were excluded from the study. In addition, 21 patients from the DN group who underwent SPK were studied longitudinally for 12 months (SPKFU). In this group, SDF and the analysis of endothelial dysfunction markers Ang-1, Ang-2 and sTM were assessed at 1 (M1), 6 (M6) and 12 (M12) months after transplantation. Mean age and duration of diabetes were similar in follow-up patients compared to patients in DN-group and SPK-group. Twenty healthy and age matched volunteers served as the control group. None of the control subjects was taking medication.

All transplantations were performed at the Leiden University Medical Center (LUMC), the Netherlands. Organs for the SPK group came from brain-dead donors registered at the national donor registry. The procedure of SPK transplantation has been described previously (23). All SPK transplants were performed through a midline abdominal incision with both organs placed intraperitoneally. The kidney was placed in the left iliac fossa, with its vessels anastomosed to the common or external iliac vessels in an end-to-side fashion. The ureter urinary bladder anastomosis was performed using the Lich–Gregoir technique. Double-J stenting of the anastomosis was used. The portal vein of the pancreas graft was anastomosed end-to-side to the recipient's vena cava inferior or right iliac vessels. The superior mesenteric and splenic arteries were reconstructed using a donor iliac artery Y graft. In some cases, the pancreas graft was procured with the celiac trunk and superior mesenteric artery on the aorta patch in which no vascular reconstruction was needed. All pancreas grafts were anastomosed end-to-side to the common iliac artery of the recipient. In 22 of the 38 patients (58%) patients exocrine pancreatic juices were drained via the bladder, followed by enteric conversion and

the other patients had a direct enteric drainage. SPK patients used at least two types of immunosuppression. Type of induction immunosuppression during transplantation was dependent on date of transplantation. Patients transplanted before 2008 received induction with interleukin-2 receptor blocker daclizumab, (100 mg/day) on the day of transplantation and 10 days after transplantation, followed by triple therapy with prednisone (tapered to a dose of 10 mg by month 3), tacrolimus or cyclosporine and mycophenolate mofetil (MMF) (target area under the curve (AUC) 30-60 ng.h/ml). Cyclosporine and tacrolimus AUC were estimated using a population based, two-compartment pharmacokinetic model combined with limited sampling and Bayesian fitting (24-26). After each AUC assessment, dosage adjustments were made to achieve the predefined target AUC: Cyclosporine AUC: 5400 ng.h/ml within the first 6 wk, which corresponds with a mean average trough level of 225 ng/ml; after 6 wk, 3250 ng.h/ml, which corresponds to a mean average trough level of 125 ng/ml; tacrolimus AUC: 210 ng.h/ml within the first 6 wk, which corresponds to a mean average trough level of 12.5 ng/ml; after 6 wk, 125 ng.h/ml, which corresponds with a mean average trough level of 7.5 ng/ml. Patients transplanted after 2008 received induction therapy with anti-CD52 antibody alemtuzumab (15 mg/day, subcutaneously) for two days followed by glucocorticoid-free therapy with MMF and tacrolimus (n=12). In the SPKFU group, twenty patients received alemtuzumab as induction therapy and one patient received basiliximab (20 mg/day) at day 0 and 4.

Patients that received a KTx were transplanted in the following way: the iliac vessels were reached through a pararectal incision. The donor's renal vessels were anastomosed end-to-side to the common or external iliac vessels of the recipient. The ureter was anastomosed to the urinary bladder using the Lich–Gregoir technique. Double-J stenting for the vesico-ureteric anastomosis was used (27). Patients received daclizumab as induction therapy after solitary KTx (100 mg/day on the day of transplantation and 10 days after transplantation) followed by triple therapy with prednisone (tapered to a dose of 10 mg by 6 weeks), tacrolimus (AUC 210 ng.h/ml first 6 weeks, then 125 ng.h/ml) or cyclosporine (AUC 5400 ng.h/ml first 6 weeks then 3250 ng.h/ml) and MMF (AUC 30-60 ng.h/ml). Patients were treated routinely with oral val-ganciclovir prophylaxis for 3 months, except for a CMV negative donor recipient status.

Microcirculatory imaging

The SDF microscan (MicroVision Medical Inc., Wallingford, PA, U.S.A) was performed as described earlier with minor modifications (22). In short, all patients were measured in supine position in a temperature controlled room (22^{o}C) by a trained observer. Oral mucosal microvasculature in the labial area was visualized with a 5x objective with a 0.2 NA providing a 325-fold magnification on screen and were sized 720 x 576 pixels (8 bit grey-scale). Ten video files (50 frames each) were obtained of capillary loops in labial mucosa from each quadrant of the lips and saved as Y800-AVI files using ICcapture imaging software (The Imaging Source Europe GmbH, Bremen, Germany). The portable, handheld device was connected

to a computer and monitor via an analog to digital converter (Domino melody framegrabber, Euresys s.a., Angleur, Belgium). A concise review about the SDF imaging technique has been described previously (21).

Analysis of SDF measurements

Before analysis, the video files were anonymized so that the assessor was blinded to the subject's identity. Capillary loops were assessed by two individual assessors in a randomized, blind fashion. From the forty video files obtained of the microcirculation, the four technically best files were selected from each quadrant of the lip for analysis. Mean vessel density (capillaries/mm^2) was calculated by observation of number of vessels per screen shot. Subsequently, tortuosity of capillary loops was assessed according to a validated scoring system described previously and the average of assessed capillary tortuosity was used to calculate mean tortuosity index per patient (22).

Laboratory assessment and endothelial structure evaluation

All persons enrolled in this study underwent routine venous blood sampling before the morning intake of immunosuppression. The following data were evaluated: creatinin, urea, HbA1c, glucose, hemoglobin and proteinuria in 24 hours urine. Glomerular filtration rate was calculated with creatinin concentration using the Modification of Diet in Renal Disease (MDRD) formula. Simultaneously, blood was collected for analysis of serum Ang-1, Ang-2 and sTM. Blood collection tubes were centrifuged for 10 minutes at 3000 rpm after which serum was stored in microcentrifuge tubes at -20^{o}C until required for analysis. Ang-1, Ang-2 and sTM concentrations were measured by enzyme-linked immuno sorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA and Diaclone Research, Besançon, France) according to the manufacturer supplied protocol. The intra-and inter-assay coefficients of variation were 3.3% and 6.4% for Ang-1, 6.5% and 9.1% for Ang-2 levels and 3.9% and 9.8%, respectively, for sTM levels. Since the Ang-2/Ang-1 ratio, rather than the absolute levels of either cytokine has been considered to determine the functional status of the microvasculature, we calculated this ratio in the different groups (44; 45).

Statistical analyses

Continuous normally distributed data are presented as mean ± standard error of the mean (SEM), unless stated otherwise. Differences between two groups in the cross-sectional study were analyzed using the unpaired two-sample T-test. When criteria for parametric testing were not met, median and interquartile range (IQR) are presented and tested with the Mann-Whitney test. Categorical variables were analyzed by a Chi-square test. In addition, multivariable linear regression was used to adjust for possible confounders. Comparisons of mean differences between the four time points in the longitudinal SPKFU study were performed using repeated measures ANOVA.

Correlations between interval variables were calculated using the Spearman rank correlation. Differences were considered statistically significant with $p<0.05$. Data analysis was performed using SPSS version 17.0 (SPSS Inc, Chicago, IL) and GraphPad Prism, version 5.0 (GraphPad Prism Software Inc, San Diego, CA).

Results

Patient characteristics

Baseline subject characteristics of DN, DM≥40ml/min, SPK and KTx groups in the cross-sectional study are presented in Table 1. There were no significant differences in patient characteristics between the DN and SPK group, with the exception of age, glucose and HbA1c levels, proteinuria and eGFR ($p<0.05$). Compared to the DM≥40ml/min patients, DN group had significantly higher systolic and diastolic blood pressure, decreased eGFR and proteinuria levels and higher HbA1c levels ($p<0.05$). As expected, SPK showed improvement in renal function and normalization in blood glucose metabolism compared to DN and DM≥40ml/min patients ($p<0.05$). The median follow up period of patients after SPK was 45.0 months (I.Q.R 18.5-106.5) and after KTx 38.5 months (I.Q.R 8.5-73.5). Patients in the KTx group had significantly better renal function compared with DN patients and higher glucose and HbA1c levels compared with SPK patients.

More than 2 years after transplantation, three patients developed diabetes type II in the SPK group of which two were treated with oral antidiabetic medication and one patient with insulin. Fourteen of the 38 (37%) patients experienced acute rejection after SPK, which is similar to rejection rates reported in other studies after SPK (28). Of these, 10 patients had a renal biopsy proven interstitial acute rejection, one of these patients had also vascular rejection. Four patients were treated as rejection, without prior transplant biopsy. Seven patients were successfully treated with solumedrol, six patients with solumedrol and anti-thymocyte globulin (ATG) and one patient with ATG alone. In the KTx group, no patients developed rejection after transplantation.

Table 1: Patient characteristics of controls, diabetes mellitus Type 1 patients with an eGFR of ≥40 ml/min (DM ≥40 ml/min), diabetic nephropathy patients (DN), simultaneous pancreas kidney transplantation (SPK) and kidney transplantation (KTx) patients.

	Controls (N=20)		DM≥40ml/min (N=15)		DN (N=26)		SPK (N=38)		KTx (N=15)	
Age (years)	44.8	± 11 [c]	54.6	± 13	43.8	± 6 [c]	48.7	± 9 [a, c]	47.6	± 10
Sex, male N (%)	10	(50%)	6	(40%)	18	(72%)	25	(66%)	6	(40%)
Smoking, N (%)	0	(0%)	2	(15%)	0	(0%)	3	(8%)	1	(7%)
Median time since transplantation (months) (IQR)	-		-		-		45.0	(18.5-106.5)	38.5	(8.5-73.5)*
Duration Diabetes (yrs)	-		35.5	± 10	29.3	± 8	27.9	± 9	34.9	± 9
BMI (kg/m²)	25.3	± 4	23.8	±3	25.2	± 3	24.4	± 4	25.0	± 5
Dialysis, N (%)	-		0	(0%)	4	(5%)	0	(0%)	0	(0%)
Systolic BP (mmHg)	135	± 18	130	± 13	145	± 20 [c]	141	± 25	138	± 29
Diastolic BP (mmHg)	83	± 9 [c]	71	± 8	86	± 11 [c]	82	± 13 [c]	81	± 14 [c]
eGFR (ml/min/1,73 m²)	92.7	± 16 [b, c, e]	69.9	± 24	23.9	± 20 [c]	52.7	± 19 [a, c]	62.0	± 23 [a]
Median proteinuria (g/24hr) (IQR)	-		0.3	(0.1-0.3)	0.7	(0.3-1.5) [c]	0.3	(0.2-0.8) [a]	0.2	(0.2-0.4) [b]
HbA1c (%)	-		7.1	± 1	8.8	± 2 [c]	5.6	± 1 [a, c]	8.5	± 1 [b,c]
Glucose (mmol/L)	5.3	± 1 [a, c, e]	12.8	± 5	13.3	± 6	5.8	± 3 [a, c]	13	± 7 [d]
Hemoglobin (mmol/L)	8.7	± 1 [b]	8.2	± 1	7.6	± 1	8.1	± 1	8.5	±1
Rejection after SPK or KTx, N (%)	-		-		-		14	(37%)	1	(7%)
Diabetes after SPK, N (%)	-		-		-		3	(8%)		
Acetylsalicylic acid, N (%)	-		4	(27%)	4	(15%)	11	(29%)	3	(20%)
Anti-hypertensives, N (%)	-									
ACE inhibitor			0	(0%)	16	(64%)	15	(40%)	7	(47%)
Diuretics			5	(33%)	13	(52%)	9	(24%) [a]	4	(27%)
β-blockers			0	(0%)	10	(40%) [c]	19	(50%) [c]	6	(40%) [c]
Calcium antagonists			2	(13%)	11	(44%) [c]	23	(61%) [c]	7	(47%) [c]
Angiotensin-II antagonists			3	(20%)	13	(52%) [c]	8	(21%) [a]	0	(0%) [a]
Statines, N (%)	-		8	(53%)	16	(60%)	27	(71%)	5	(33%) [d]
Steroid-free, Alemtuzumab induction, N (%)	-		-		-		12	(13%)	-	
Immunosuppressive, N (%)	-		-		-					
Cyclosporine							13	(34%)	1	(7%) [d]
Tacrolimus							25	(66%)	12	(80%)
Prednisone							27	(71%)	9	(60%)
Azathioprine							3	(8%)	0	(0%)
Everolimus							2	(5%)	0	(0%)
Sirolimus							0	(0%)	1	(7%)
Mycophenolate Mofetil							28	(74%)	14	(93%)

All data are mean ±SD, unless otherwise specified. * The patients in this group who received SPK lost their pancreas graft after 2 and 4 days following transplantation (n=2).
[a] p<0.05 vs DN group [b] p<0.05 vs DN and SPK group [c] p<0.05 vs DM≥40 ml/min [d] p<0.05 vs SPK [e] p<0.05 vs KTX.
BMI, body mass index; BP, blood pressure; ACE, angiotensin converting enzyme; eGFR,estimated glomerular filtration rate; IQR, interquartile range; SPK, simultaneous pancreas kidney transplantation;

Table 2: Patient characteristics in SPKFU patients at 1 (M1), 6 (M6) and 12 (M12) months after SPK transplantation.

Characteristic	M1** (N=21)		M6 (N=21)		M12 (N=21)	
Age (yrs)	44.0	± 6	44.4	± 6	45.0	± 6
Sex, male N (%)	16	(76%)	-		-	
Smoking, N (%)	0		0		0	
BMI (kg/m^2)	23.8*	± 3	24.0	± 2	24.1	± 3*
Dialysis, N (%)	0		0		0	
Systolic BP (mmHg)	123	± 22	132	± 23	130	± 16
Diastolic BP (mmHg)	75	± 13	777	± 13	78	± 6
eGFR (ml/min/1,73 m^2)	56.5	± 23 *	55.2	± 15 *	54.1	± 10 *
Median proteinuria (g/24hr) (IQR)	0.3	(0.3-1.2)	0.3	(0.2-1.0)	0.3	(0.1-0.7)
HbA1c (%)	6.7	± 2	5.3	± 0 *	5.4	± 0 *
Glucose (mmol/L)	6.2	± 1 *	5.3	± 1 *	5.7	± 1 *
Hemoglobin (mmol/L)	6.6	± 1	7.4	± 1	7.9	± 1
Rejection after transplantation, N (%)	0		3	(14%)	2	(10%)
Diabetes after SPK, N (%)	1	(5%)	2	(10%)	0	
Acetylsalicylic acid, N (%)	3	(14%)	1	(5%)	4	(19%)
Anti-hypertensives, N (%)						
ACE inhibitor	2	(10%)	3	(14%)	4	(19%)
Diuretics	0	(0%)	1	(5%)	2	(10%)
β-blockers	7	(33%)	4	(19%)	4	(19%)
Calcium antagonists	4	(19%)	5	(24%)	8	(38%)
Angiotensin-II antagonists	0		0		0	
Statines, N (%)	2	(10%)	2	(10%)	3	(14%)
Steroid-free, Alemtuzumab induction, N (%)	20	(95%)	-		-	
Immunosuppressive, N (%)						
Tacrolimus	18	(86%)	17	(81%)	16	(76%)
Ciclosporine	2	(5%)	3	(14%)	3	(14%)
Prednisone	1	(5%)	6	(29%)	6	(29%)
Mycophenolate Mofetil	21	(100%)	20	(95%)	20	(95%)
Everolimus	1	(5%)	1	(5%)	2	(10%)

All data are mean ±SD, unless otherwise specified. * p<0.05 vs DN from Table 1. **Patients were recruited from the DN group (see Table 1 for patient characteristics). BMI, body mass index; BP, blood pressure; ACE, angiotensin converting enzyme; eGFR, estimated glomerular filtration rate; IQR, interquartile range; SPK, simultaneous pancreas kidney transplantation;

The clinical characteristics of the patients in the longitudinal SPKFU group are shown in Table 2. As expected, patients showed normalization of glucose levels (p=0.04) and eGFR (p=0.006) at 1 month after SPK. Since all the patients in the SPKFU group were transplanted after 2008, immunosuppressive therapy was initially glucocorticosteroid-free, with MMF and tacrolimus as maintenance therapy. Twelve months after SPK, two patients were converted to everolimus due to side effects of tacrolimus. In four patients treatment with prednisone was added at the time of conversion from tacrolimus to cyclosporine due to side effects caused by

tacrolimus (2 patients) or MMF (2 patients). In this group, five patients experienced interstitial rejection and one patient had also vascular rejection after transplantation. These patients were treated with prednisolone and ATG. Six month after SPK, 2 patient developed diabetes type II, which was treated with oral antidiabetic medication.

Reversibility in capillary tortuosity after SPK

There was no difference in the capillary density in the cross-sectional study between the DN (mean 19.88 ±4.1, SEM), DM≥40ml/min (mean 19.03 ±0.5, SEM), SPK (mean 20.79 ±3.1, SEM), control (mean 21.34 ±3.1, SEM) and KTx (mean 19.14 ±0.5, SEM) group. However, the morphology of the capillaries in the DN and DM≥40ml/min patients was significantly disturbed compared to the controls (Fig 1A). We observed significantly more capillary tortuosity in the DN patients (mean 1.83 ±0.4, SEM, $p<0.001$) and DM≥40ml/min patients (mean 1.55 ±0.1, SEM, $p<0.0001$) compared to the controls (mean 1.15 ±0.2, SEM). After KTx (mean 1.64 ±0.1, SEM) there was no significant decrease in capillary tortuosity compared to DN ($p=0.06$) and DM≥40ml/min ($p=0.3656$) group. However, interestingly, SPK (mean 1.31 ± 0.3, SEM, $p<0.001$) showed reversal in capillary tortuosity compared to DN and DM≥40ml/min group (Fig 1B). The three patients who developed diabetes type II after SPK did not show more increased capillary tortuosity compared to the other patients in the SPK group.

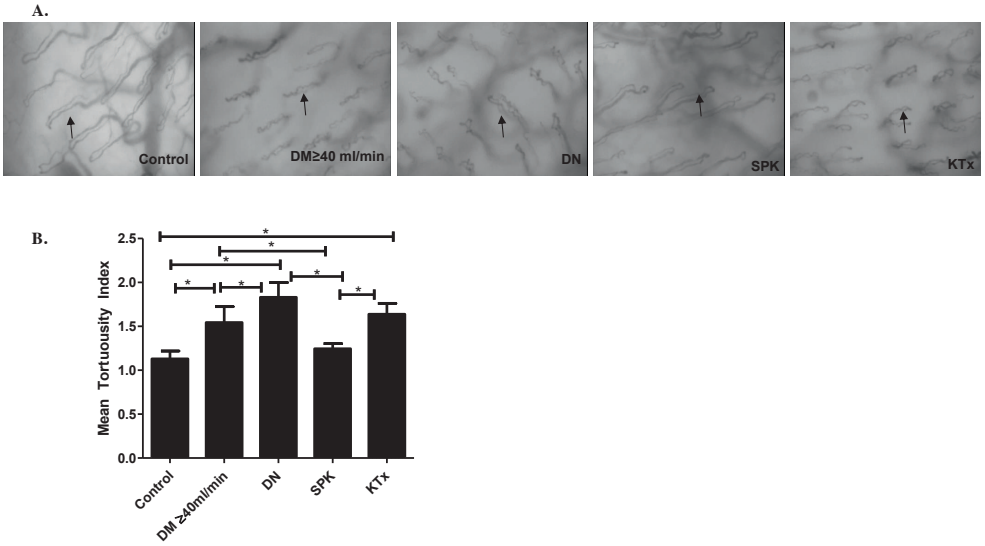

Figure 1. A. Sidestream darkfield images of the oral mucosa visualizing the microvascular capillaries of a representative patient in the control, DM≥40ml/min, DN, SPK and KTx group. Black arrows: capillary loops. B. Mean tortuosity index of microvascular capillaries in the control (n=20), DM≥40ml/min (n=15), DN (n=26), SPK (n=38) and KTx (n=15) group. Data shown are mean±SEM. *P<0.05.

Next, we investigated capillary density and morphology longitudinally in the SPKFU group before, 1, 6 and 12 months after SPK. No difference was observed in capillary density before and after SPK. Most importantly, SPK showed reversal of capillary tortuosity within 1 year after SPK, reaching significance at 6 and 12 months after transplantation (mean 1.52 ±0.1, SEM and 1.23 ±0.0 versus DN 1.83 ±0.4, p<0.01 and p<0.001, respectively) (Fig 2AB). The differences remained significant in both the cross-sectional and SPKFU group after adjustment for age, sex, BMI body mass index (BMI), systolic and diastolic blood pressure and smoking.

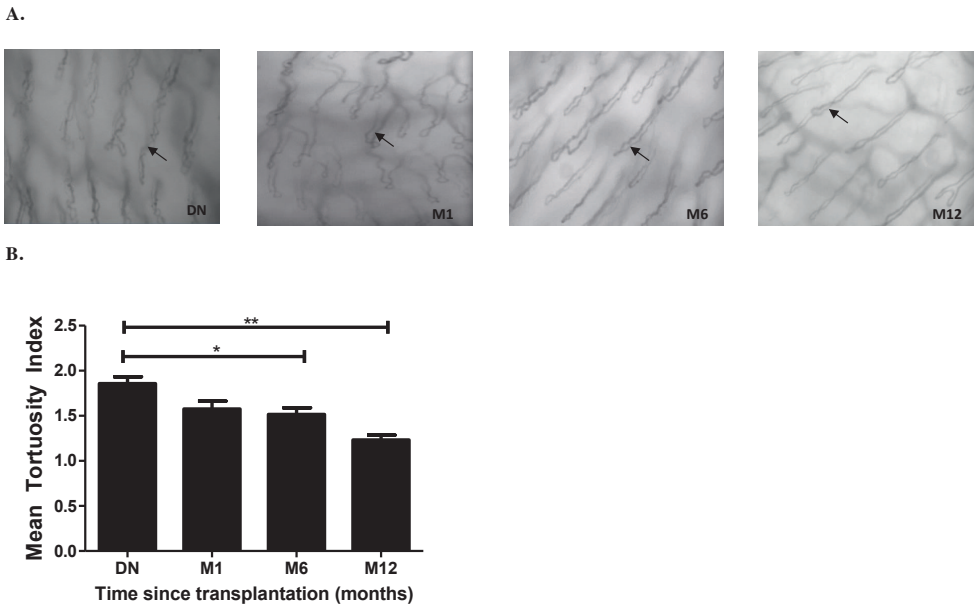

Figure 2. A. Sidestream dark field images of the oral mucosa visualizing the microvascular capillaries of a representative DN patient before (DN), 1 (M1), 6 (M6) and 12 (M12) months after SPK in the longitudinal SPKFU group. Black arrows: capillary loops. B. Longitudinal course of the mean tortuosity index of microvascular capillaries in DN patients before (DN,=n=26), 1 (M1, n=21), 6 (M6, n=21) and 12 (M12, n=21) months after SPK in the SPKFU group. Data shown are mean±SEM. *P<0.01, M6 compared to DN.**P<0.001, M12 compared to DN.

Correlation between tortuosity, Hb1Ac, renal function and previous rejection

Next, correlation analyses in the SPK patients was performed for the capillary density and tortuosity index with several factors that are known to influence the microvasculature, including time since transplantation, eGFR, HbA1c, proteinuria, and previous rejection. No correlation was found between capillary density and time since transplantation, eGFR, HbA1c, proteinuria and previous rejection. There was a significant correlation between tortuosity with eGFR (r =-0.26, p= 0.005), Hb1Ac levels (r =0.40, p<0.0001) and previous rejection (p= 0.0160). There was no association between tortuosity index and time since transplantation or proteinuria (data not shown).

Reversal to baseline of Ang-2/Ang-1 ratio after SPK

To evaluate the effects of SPK on the microvascular conditions, we also measured serum levels of sTM, Ang-1 and Ang-2 and calculated the Ang-2/Ang-1 ratio as markers for endothelial dysfunction. We detected a significant decrease in sTM levels in patients after SPK (mean 9.7 ±1 ng/ml, SEM, p=0.004) and KTx (mean 6.3 ±1 ng/ml, SEM, p<0.0001) compared to DN patients (mean 15.2 ±1 ng/ml, SEM, p=0.004) (Fig 3A). In the SPKFU group, sTM levels started to decrease at 1 month (mean 13.35 ±2.0 ng/ml, SEM) after transplantation and remained low after 6 months (mean 11.83 ±1.6 ng/ml, SEM), reaching significance at 12 months (10.42 ±1.2 ng/ml, SEM) as compared to before SPK (mean 15.2 ±1 ng/ml, SEM, p<0.01) (Fig 3B). In addition, our results demonstrate normalization in Ang-2/Ang-1 ratio after SPK (mean 0.08 ±0.02, SEM) compared to the DN (mean 0.16 ±0.04, SEM, p=0.04) and DM≥40ml/min patients (mean 0.16 ±0.04, SEM, p=0.03), but no significant decrease after KTx (mean 0.20 ±0.04, SEM, p=0.6219 and p=0.5437, respectively) (Fig 3C). In the longitudinal study, the Ang-2/Ang-1 ratio started to show a decrease at 6 months (mean 0.09 ±0.03, SEM) after SPK and remained decreased at 12 months (mean 0.09 ±0.02, SEM) following transplantation compared to before SPK (mean 0.16 ±0.04, SEM), however, statistical significance was not reached (p=0.13 and p=0.14, respectively) (data not shown).

Correlation of endothelial function markers with capillary tortuosity, renal function and HbA1c

Next, the correlation between the different endothelial dysfunction markers, capillary density and morphology, eGFR, HbA1c, previous rejection and time since transplantation was analyzed. No correlation between endothelial dysfunction markers and capillary density or tortuosity was found. Moreover, there was no correlation between time since transplantation, previous rejection and endothelial dysfunction markers. Increased Ang-2 and sTM levels correlated significantly with decreased eGFR (r= -0.53, p<0.0001 and r=-0.28, p=0.0083) and high Hb1Ac levels with increased Ang-2 (r= 0.23, p=0.0319). Interestingly, Ang-2/Ang-1 ratio correlated significantly with capillary tortuosity (r=0.21, p=0.0348), HbA1c (r=0.32, p=0.0038), eGFR (r=-0.29, p=0.0460) and proteinuria levels (r=0.2640, p=0.0365) (data not shown).

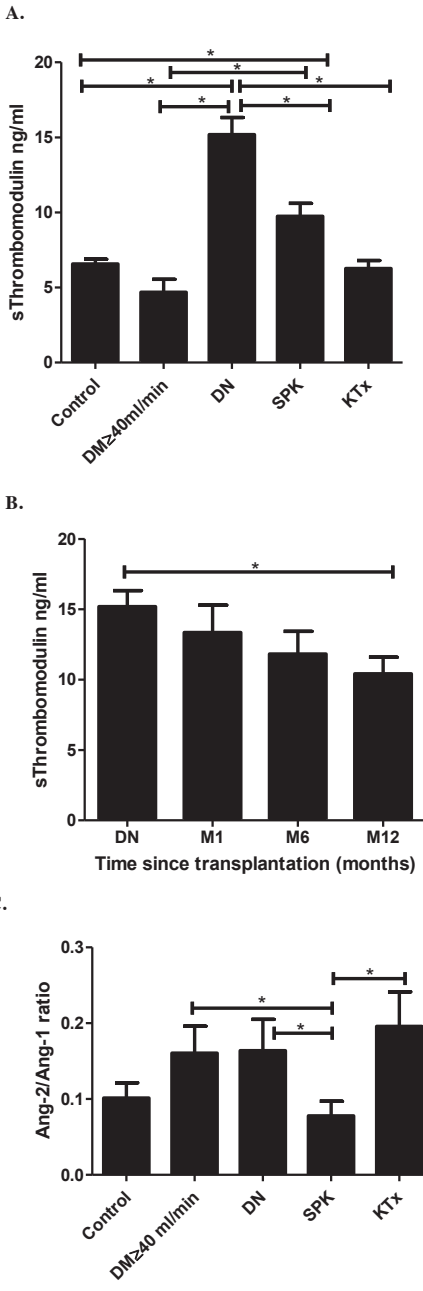

Figure 3. A. Soluble thrombomodulin serum levels in the control (n=20), DM≥40ml/min (n=15), DN (n=26), SPK (n=38) and KTx (n=15) group. *P<0.05. B. Soluble thrombomodulin serum levels in DN patients before (DN), 1 (M1), 6 (M6) and 12 (M12) months after SPK in the SPKFU group. *P<0.05 compared to DN. C.Ang-2/ Ang-1 ratio in the control (n=20), DM≥40ml/min (n=15), DN (n=26), SPK (n=38) and KTx (n=15) group. Data shown are mean±SEM. *P<0.05.

Discussion

This study shows increased microvascular tortuosity using SDF imaging, and a dysbalance in Ang-2/Ang-1 ratio in DM type 1 patients. Interestingly, we demonstrated reversal of capillary tortuosity and normalization of the Ang-2/Ang-1 ratio after SPK, but not after KTx alone. Furthermore, increased microvascular tortuosity correlated with increased Ang-2/Ang-1 ratio. Importantly, the longitudinal study demonstrated that both reversibility of microvascular damage and decrease in sTM and Ang-2/Ang-1 balance occurred in the first year after SPK. These findings suggest that SPK is effective in reversing systemic microvascular complications in DN patients early after transplantation. Assessment of capillary tortuosity, as marker for microvascular disease, using the non-invasive SDF imaging can be used to estimate the degree of microvascular derangements in DN patients before and after SPK.
Microvascular disease is one of the most important drivers of diabetic complications (29;30). Several mechanisms have been described for the pathogenesis of microvascular disorders in patients with diabetes mellitus. The capillaries in diabetic patients tend to be leaky and tortuous, lacking the hierarchical arrangement of arteries, capillaries and venules due to perivascular stromal cell loss (31-33). Hyperglycemia in particular can initiate disturbances in blood flow and afflict the interaction between perivascular stromal cells and microvascular endothelial cells. Hereby, the loss of perivascular stromal cells leads to loss of its primary functions, including autoregulation of the vessel integrity and compensatory mechanisms for the fluctuating hydrostatic pressure. Specifically, this impaired auto regulation of the microvasculature could result in disruption of the basement membrane and the failure to maintain the stability of the vessel wall against irregular longitudinal traction and transmural pressure leading to dilated and tortuous vessels (32-38). Ang-2 has been assumed to destabilize vessels by promoting the weakening of perivascular stromal - endothelial cell interaction (39;40).

The increased microvascular tortuosity in diabetes as observed in the present study concurs with previous data on microvascular alterations in diabetic patients as determined by skin capillaroscopy (41). Likewise, previous clinical observations using conventional capillaroscopy revealed that diabetic retinopathy appeared to be associated with increased tortuosity as well, suggesting that this might be an early sign of microvascular damage in diabetes (33). However, compared to conventional capillaroscopy, SDF imaging has the advantage to assess the microvasculature without injecting fluorescent dyes and it enables measurement of the superficial skin and mucous microcirculation in a noninvasive manner (21). We previously could demonstrate, using SDF measurements, that the presence of microvascular tortuosity is associated with macrovascular disease in diabetics (22). However, the extensiveness of microvascular abnormalities has not been measured in DN patients before and after SPK using SDF imaging and correlation with markers for endothelial dysfunction and clinical features has not been performed yet.

Previous clinical studies showed that 10 years of normoglycemia after pancreas transplantation ameliorated the glomerular and tubular lesions that characterize diabetic nephropathy in patients with long-term type 1 diabetes who did not receive renal grafts (42). Additional studies provided evidence for improvement of diabetic polyneuropathy after pancreatic transplantation as well (43;44). Although we observed reversal of microvascular tortuosity after SPK, this study did not explore the mechanisms behind these improvements.

Increased microvascular tortuosity in DN coincided with increased levels of sTM and a disturbed Ang-2/Ang-1 balance, which is in line with previous studies that have demonstrated a positive correlation with microvascular destabilization and these markers of endothelial dysfunction (45;46). Ang-2 is a competitive ligand for the same Tie-2 receptor as for Ang-1, with competing, antagonistic effects on angiogenesis and microvascular remodeling. Increased Ang-2/Ang-1 ratio has been shown to be associated with hyperglycemia, chronic kidney disease, acute coronary syndrome, sepsis and variety of diseases known for their common characteristic of endothelial dysfunction and microvascular inflammation (45;46). The Ang-2/ Ang-1 ratio showed only normalization after SPK, while it was still increased in the presence of diabetes and also was not affected by kidney transplantation alone. In addition, the Ang-2/Ang-1 ratio correlated to capillary tortuosity underscoring the systemic nature of microvascular destabilization in diabetes. (4;5;47-50). (51).
The strength of our study was the use of a noninvasive tool to visualize the microvasculature in a study of SPK and DN patients before and after transplantation. However, our study has also some limitations. In this studied cohort there were no patients who received a solitary kidney transplant that could be followed longitudinally. Therefore, future longitudinal studies should possibly also include such patients to further corroborate the potential contribution of reversal of diabetes on the microcirculation.

In conclusion, the present study revealed a disturbed microvascular morphology in DM type 1 patients and SPK resulted in reversal of systemic microvascular derangements and normalization of markers for endothelial dysfunction. The use of SDF imaging allows for easy non-invasive and sequential monitoring of microvascular disease in patients with diabetes.

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